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IMPROVEMENTS IN OR RELATING TO ORGANIC COMPOUNDS

The present invention relates to a method for reducing the incidence of insect resistance to insecticidal plants. In particular it relates to preventing resistance of insects to transgenic insecticidal plants such as cotton from being spread through an insect population.

Millions of hectares of crops worldwide are damaged each year as a result of insect pests. Controlling insect pests is a serious problem for farmers who look to minimise such crop damage and resulting yield losses. This is especially true in cotton, a crop of great commercial importance. Thousands of hectares of cotton crops are damaged by insect pests each year.

Entomologists calculate that crop damage caused by insects has doubled in the last 50 years, related to intensified farming efforts to feed a growing world population. The agrochemical industry has tried to control this problem with chemical solutions and insecticide spraying has become a commonplace method adopted to minimise the damage to crops. Today there are over 200 different active ingredients, in some 40,000 commercial chemical products, all targeted at reducing insect damage.

During one growing season, many insecticide sprays may be applied to a single crop. This intensive use of chemical insecticides imposes a high selection pressure and can lead to the rapid build-up of resistance. Examples of insect resistance to pesticides have been documented worldwide. It is estimated that more than 500 arthropod pests worldwide have developed resistance to chemicals. In China, cotton yields fell by one-third between 1991 and 1993, largely due to cotton bollworm (*Helicoverpa armigera*) resistance to the chemicals which were used for insect control.

Insects have an exceptional ability to adapt to their environment. Genetics is the basis for the rapid build-up of resistance in many insects. Natural selection allows the insects with resistance genes to survive, and the resistance trait is passed on to their offspring. Resistant insects continue to multiply as susceptible insects are eliminated by the pesticide, until eventually, insects that have the resistance genes are predominant and the

pesticide is no longer effective. The speed with which resistance develops depends on many factors such as the rate of insect reproduction, the migration and host range of the insect, the persistence of the pesticide and how often the pesticide is applied.

There are a number of known mechanisms of resistance in insects. For example, resistant insects may naturally detoxify the toxin or remove it from their bodies faster than susceptible insects (metabolic resistance); the site where the toxin usually binds in the insect may be modified to reduce its effects (altered target-site resistance); resistant insects may absorb the toxin slower than susceptible insects (penetration resistance); or resistant insects may detect and avoid the toxin (behavioural resistance). Pests often use more than one of these mechanisms at the same time.

One solution aimed at reducing the number of insecticide sprays and managing insect resistance is to engineer the crop plants to synthesise their own insecticide. Plants may be engineered to contain, for example, insecticidal genes from other organisms. Currently the most economically significant insecticidal transgenic plants are those which contain genes from the bacterium *Bacillus thuringiensis* that produces a protein that controls Lepidopteran pests. Not only can transgenic crops reduce the use of broad-spectrum insecticides, but they also are more target-pest specific. This means populations of beneficial insects may not be affected. Pest control is easier with transgenic crops because the insecticidal toxin can be targeted to different parts of the plant, such as the roots, which are hard to spray with conventional pesticides.

However as transgenic plants provide continuous selection pressure on pest populations, there is a greater potential for resistance development than with conventional insecticides. Also, unlike chemical insecticides, there are still very few genes and proteins that are known to be effective for the protection of transgenic crops against insects. Therefore management of resistance build-up in populations of target insects is very important.

Insect Resistance Management (IRM) programs are designed to control such a build-up of resistance, and include the use of synthetic insecticides, biological insecticides,

transgenic plants, beneficial insects, cultural practices, crop rotation, pest-resistant crop varieties and chemical attractants or deterrents.

One strategy employed in IRM programs is the use of refuges (refugia). A refuge is the designation of a percentage of the crop as non-transgenic with no or reduced control treatments. Refuge areas may be within fields of transgenic or treated crops, around the border of such fields or even in adjacent fields. Refugia serve to maintain a population of pests that are susceptible to insects. When members of the susceptible population mate with any resistant insects that emerge from protected fields, their susceptible genes dilute any resistant genes in the overall population. It is thought that in certain situations, as with transgenic crops, providing such a haven for pest insects can delay or even prevent the onset of resistance.

In essence, the use of refugia serves as a mechanism for producing a population of insects pests, pests which the growers are trying to control in their field. Resistant insects which survive the insecticidal effects of feeding on the transgenic crop are not killed. Instead, the refuge provides a source of susceptible insects with which resistant insects can mate. This is a potential problem because it may actually serve to spread the resistant trait rather than contain it.

The present patent application describes a solution to this problem. The invention uses a region similar to a refuge, but comprising plants which produce at least one insecticidal toxin, said toxin being different to the toxin produced by the principal crop plants. The effect of the different insecticidal toxins is to kill insects which are resistant to the toxin of the principal crop plants rather than allowing them to breed and thus spread the resistance trait. In this way a very high selection pressure exists and insects are unlikely to survive as this would require resistance to at least two different insecticidal toxins preferably having different modes of action. Therefore the incidence of resistance is reduced. This invention may be used in conjunction with other IRM techniques.

According to the present invention there is provided a locus at which insects feed comprising at least two regions, characterised in that: a) a first region comprises plants which produce at least a first insecticidal toxin; and b) a second region comprises plants

which produce at least a second insecticidal toxin; wherein an insect which can develop resistance to the first toxin does not develop resistance to the second toxin, and the first region comprises plants which produce the first toxin but not the second toxin when the plants of the second region produce the second toxin but not the first toxin.

The word insecticidal as used herein describes the effect of a toxin on insects. It is not limited to death of the insect, but also includes any effect which is detrimental to the insect, for example sickness, anti-feedant, growth retardation and reduced fecundity.

Resistant insects are those that do not suffer any substantial or appreciable detrimental effects as a result of exposure to or ingestion of a suitable dose in insecticidal toxin. A suitable dose of insecticidal toxin may be measured by exposure to or ingestion of the toxin by a susceptible insect and identification of the dose at which detrimental effect(s) are observed. Detrimental effects to the insect are described above in the definition of the word insecticidal. These detrimental effects will reduce the incidence of transfer of a resistance trait from a resistant insect to future generations of insects.

The word plants as used herein refers to plants and plant parts and includes seeds.

The invention includes plants which produce more than one toxin, for example via gene stacking. The plants of either the first and/or second region may even produce the same toxins, with the *proviso* that the first region comprises plants which produce the first toxin but not the second toxin when the plants of the second region produce the second toxin but not the first toxin. The invention is not limited to loci which comprise first and second regions that only comprise plants which produce insecticidal toxin(s), but may also contain other plants in addition. In one aspect of the invention, the plants of either region may also produce toxins to make them resistant to non-insect pests such as viruses, fungi or nematodes. In another aspect of the invention, the plants of either region may be tolerant to chemical herbicides. In a further aspect of the invention, the locus may comprise more than two regions, wherein said additional regions may comprise plants which produce insecticidal toxins.

The skilled man will be familiar with insects which feed at the locus. Preferably, insects which feed at the locus includes pest insects which cause damage to plants. More preferably, this includes insects which are, or can develop to be, resistant to an insecticidal toxin. More preferably still, it includes insects selected from the group comprising: *Acanthoscelides obtectus*, *Bruchus sps.*, *Callosobruchus sps.* (bruchid beetles), *Agriotes sps.* (wireworms), *Amphimallon sps.* (chafer beetles), *Anthonomus grandis* (cotton boll weevil), *Ceutorhynchus assimilis* (cabbage seed weevil), *Cylas sps.* (sweet potato weevils), *Diabrotica sps.* (corn root worms), *Epicauta sps.* (black blister beetles), *Epilachna sps.* (melon beetles etc.), *Leptinotarsa decemlineata* (Colorado potato beetle) *Meligisthes sps.* (blossom beetles), *Melolontha sps.* (cockchafers), *Phyleotreta sps.*, *Psylliodes sps.* (flea beetles), *Popillia japonica* (Japanese beetle), *Scolytus sps.* (bark beetles), *Sitophilus sps.* (grain weevils), *Tenebrio molitor* (yellow mealworm), *Tribolium sps.* (flour beetles), *Trogoderma granarium* (Khapra beetle), *Acleris sps.* (fruit tree tortrixs), *Acraea acerata* (sweet potato butterfly), *Agrotis sps.* (cutworms), *Autographa gamma* (silver-Y moth), *Chilo sps.* (stalk borers), *Cydia pomonella* (codling moth), *Diparopsis sps.* (red bollworms), *Ephestia sps.* (warehouse moths), *Heliothis sps.*, *Helicoverpa sps.* (budworms, bollworms), *Mamestra brassicae* (cabbage moth), *Manduca sps.* (hornworms), *Maruca testulalis* (mung moth), *Mythimna sps.* (cereal armyworms), *Ostrinia nubilalis* (European corn borer), *Pectinophora gossypiella* (pink bollworm), *Phthorimaea operculella* (potato tuber moth), *Pieris brassicae* (large white butterfly), *Pieris rapae* (small white butterfly), *Plodia interpunctella* (Indian grain moth), *Plutella xylostella* (diamond-back moth), *Pseudoplusia includens* (soybean looper), *Sitatroga cerealella* (Angoumois grain moth), *Spodoptera sps.* (armyworms), *Trichoplusia ni* (cabbage semilooper), *Acheta sps.* (field crickets), *Gryllotalph sps.* (mole crickets), *Locusta migratoria* (migratory locust), *Schistocerca gregaria* (desert locust), *Acrythosiphon pisum*, *Drosophila sp.*, *Acrosternum hilare* (green stink bug), *Aphis gossypii* (cotton aphid), *Campylomma liebnechti* (apple dimpling bug), *Creontiades dilutus* (green mirid), *Crocidosema plebejana* (cotton tipworm), Earias huegelli (rough bollworm), *Euschistus servus* (brown stink bug), *Frankliniella sps.* (thrips), *Lygus lineolaris* (tarnished plant bug), *Tetranychus urticae* (spider mite) and *Thrips tabaci* (onion thrips).

Most preferably, it includes insects selected from the group comprising: *Anthonomus grandis* (cotton boll weevil), *Pectinophora gossypiella* (pink bollworm), *Heliothis virescens* (tobacco budworm), *Helicoverpa zea* (cotton bollworm), *Helicoverpa armigera* (American bollworm), *Helicoverpa punctigera* (native bollworm), *Spodoptera exigua* (beet armyworm) and *Spodoptera frugiperda* (fall armyworm).

With the benefit of the present disclosure, the skilled man will be familiar with insecticidal toxins that can be expressed in plants which may be suitable for use in this invention. Suitable toxins may even be those known in the prior art. For example, they include crystal proteins from *Bacillus thuringiensis*, many of which are have been extensively studied and are well known in the prior art such as Cry1Ac, Cry2Ab and Cry1F. Further non-limiting examples of insecticidal toxins are vegetative insecticidal proteins VIP3A and VIP3B from *Bacillus thuringiensis*, 445 from *Paecilomyces farinosus* (see International Patent Application publication number WO01/00841) and GGK (see International Patent Application publication number WO02/098911). Alternative suitable insecticidal toxins may, for example, be isolated from bacteria, fungi, plants or other sources. The genes encoding these toxins can be cloned and transformed into suitable plants under the control of a plant-operable gene cassette, using standard molecular and cell biology techniques. The toxins may be targeted to particular parts of the plant such as the roots, leaves or seeds by cloning the genes encoding the toxins to be under the control of tissue-specific promoters. Alternatively, the toxins may only be produced at a certain growth stage of the plant through use of inducible or temporal promoters. The toxins first and second insecticidal toxins may be insecticidal towards different spectra of insect species. Preferably the first and second toxins are insecticidal towards the same or similar insect species, or overlapping spectra of insect species.

In one aspect of the invention, the plants of the first and second regions may optionally exhibit other beneficial traits, which also may have been introduced via gene cloning and plant transformation. Any number of these traits may be stacked with the insecticidal toxin in the plants. For example, the plants of either region may exhibit resistance to a particular herbicide, fungal disease, viral infection or nematode infestation. An example of resistance to a herbicide is described in International Patent Application publication

number WO 00/66747 wherein a mutant form of the enzyme EPSP synthase is expressed in a plant so that the plant is tolerant to the herbicide glyphosate.

In a particular embodiment of the present invention, the second region is within a quarter of a mile from the first region. In a further embodiment the second region is adjacent to the first region. In a still further embodiment the second region is a border around the perimeter of the first region. In a further embodiment the second region comprises one or more strips within the first region. In a still further embodiment a plurality of first and second regions are present in a mosaic pattern. In a further embodiment the locus comprises a random distribution of first and second regions within the locus. The schematic diagrams provided in Figures 1 to 10 represent non-limiting examples of the possible arrangement of first and second regions within the locus of the present invention.

A locus according to the present invention may be comprised by a farm, wherein the at least two regions are fields. The following non-limiting examples describe possible arrangements of the fields. For example, the fields may be adjacent to one another (see Figures 5 to 7). Alternatively, the locus of the present invention may be a field, wherein the at least two regions are areas of the field comprising different plants. The second region may be arranged, for example, as a border around the perimeter of the first region (see Figure 1), as a series of horizontal or vertical strips amongst the first regions (see Figures 2 and 3) or as a block within the first region (see Figure 4). A plurality of first and second regions may be present in a mosaic pattern, for example as depicted in Figure 8, 9 or 10. Plants of the first and second regions may be interplanted, or distributed randomly within the locus. In one aspect of the invention, there may be a border around the locus comprising or consisting of non-host plants or being uncultivated so that insects cannot migrate outside of the locus.

Alternatively, a locus according to the present invention may be comprised by, for example, a garden, forest, glasshouse or seed store. The locus may even be comprised by a lake such that the invention is used to control aquatic insects. However, the scope of this invention is clearly restricted to loci wherein the invention would be functional. The skilled man would understand that the present invention excludes the possibility of the

locus being the world, wherein the first region is America and the second region is Europe, for example.

In a particular embodiment of the present invention the locus comprises at least two regions wherein the first region comprises plants which produce at least a first insecticidal toxin, and the second region comprises plants which produce at least a second insecticidal toxin, wherein the first insecticidal toxin has a different mode of action to the second insecticidal toxin. Examples of known modes of action of insecticidal chemicals and toxins include, but are not limited to acetyl choline esterase inhibitors, GABA-gated chloride channel antagonists, sodium channel modulators, acetyl choline receptor modulators, chloride channel activators, juvenile hormone mimics, fumigants, selective feeding blockers, growth inhibitors, disrupters of insect midgut membranes, inhibitors or oxidative phosphorylation, disrupters of ATP formation, oxidative phosphorylation uncouplers, inhibitors of magnesium stimulated ATPase, inhibitors of chitin biosynthesis, ecdysone agonist or disrupters, electron transport inhibitors and voltage dependant sodium channel blockers. For example, the first toxin may be a disrupter of the insect midgut membrane, such as a crystal toxin from *Bacillus thuringiensis*, and the second toxin may be a growth inhibitor.

In a further embodiment of the invention, the first insecticidal toxin is a crystal protein from *Bacillus thuringiensis* and the second insecticidal toxin is a vegetative insecticidal protein (VIP) from *Bacillus thuringiensis*, or vice versa. Many crystal proteins from *Bacillus thuringiensis* have been isolated and are known to have an insecticidal effect. Preferably, the crystal protein of the present invention is Cry1Ac. Preferably the VIP protein is VIP3A. In one aspect of the invention, the plants which produce either the first or second insecticidal toxin may comprise both Cry1Ac and Cry2Ab.

In a further embodiment of the present invention, the plants which comprise the first toxin and the plants which comprise the second toxin are from different genera. For example, the plants which comprise the first toxin may be cotton plants from the genus *Gossypium* L., and the plants which comprise the second toxin may be corn plants from the genus *Zea* L.. In further non-limiting example, the plants which comprise the first

toxin may be wheat plants from the genus *Triticum* L. and the plants which comprise the second toxin may be barley plants from the genus *Hordeum* L..

In a further embodiment of the present invention, the plants which comprise the first toxin and the plants which comprise the second toxin are cotton plants. Preferably the plants are of the same genus. More preferably the plants are the Upland Cotton species, *Gossypium hirsutum*. The cotton plants of the first and second regions may be different varieties.

In a further embodiment of the invention, at least 5% of the locus comprises the first region and at least 5% of the locus comprises the second region. Preferably, at least 20% of the locus comprises the first region and at least 20% of the locus comprises the second region. More preferably, 50% of the locus comprises the first region and 50% of the locus comprises the second region.

Further, the invention provides the option of applying a chemical spray to some or all of the regions or parts of said regions within the locus. The chemical may, for example, be an insecticide, fungicide or herbicide. Preferably such an insecticidal chemical has a different mode of action to the insecticidal toxins produced by the plants of the first and / or second regions. More preferably, the chemical is not a *Bacillus thuringiensis* insecticide.

According to the present invention there is provided a method of controlling insects comprising providing a locus at which insects feed comprising at least two regions, characterised in that: a) a first region comprises plants which produce at least a first insecticidal toxin; and b) a second region comprises plants which produce at least a second insecticidal toxin; wherein an insect which can develop resistance to the first toxin does not develop resistance to the second toxin, and the first region comprises plants which produce the first toxin but not the second toxin when the plants of the second region produce the second toxin but not the first toxin.

The words 'controlling' or 'control' as used herein refer not just to death of insects, but also include other detrimental effects on insects such as sickness, anti-feedant, growth retardation and reduced fecundity.

In an embodiment of the invention, a locus as described above is used in a method of controlling insects.

According to the present invention there is provided a method of reducing the incidence of resistance to a first insecticidal toxin comprising the steps of providing a locus at which insects feed comprising at least two regions, characterised in that: a) a first region comprises plants which produce at least a first insecticidal toxin; and b) a second region comprises plants which produce at least a second insecticidal toxin; wherein an insect which can develop resistance to the first toxin does not develop resistance to the second toxin, and the first region comprises plants which produce the first toxin but not the second when the plants of the second region produce the second toxin but not the first, so that insects which have developed or are developing resistance to the first insecticidal toxin are controlled by the second toxin.

The terms 'developed resistance' and 'developing resistance' refer to resistance within a population of insects rather than individual insects. While it may be possible for an individual insect to become resistant or more resistant to an insecticidal toxin, for example by the overproduction of a detoxification enzyme in response to ingestion of the toxin, resistance is more likely to occur as a result of one or more mutations in the insect genome. In this way, insects are either born resistant or susceptible to a particular toxin. Therefore insects which have 'developed' or are 'developing resistance' encompasses the development of resistance via evolution through generations of breeding.

In an embodiment of the invention, a locus as described above is used in a method of reducing the incidence of resistance of insects to a first insecticidal toxin.

In a further embodiment of the present invention, there is a method as described above or a locus as described above wherein either the first or second region comprises Bollgard® cotton plants. Preferably either the first or second region comprises Bollgard I® cotton

plants which produce the insecticidal toxin Cry1Ac. More preferably either the first or second region comprises Bollgard II® cotton plants which produce the insecticidal toxins Cry1Ac and Cry2Ab in the same plant.

In a further embodiment of the present invention, there is a method as described above or a locus as described above wherein the first region comprises Bollgard® cotton plants and the second region comprises VIP cotton plants. In a further embodiment still, there is a method as described above or a locus as described above wherein the first region comprises VIP cotton plants and the second region comprises Bollgard® cotton plants. The Bollgard® cotton plants may be Bollgard I® or Bollgard II®. The VIP cotton plants produce the insecticidal toxin VIP3A (see, for example, British Patent Application Number GB0225129.6). In a further aspect of the invention, the first region comprises VIP cotton plants and the second region comprises Bollgard® cotton plants.

DESCRIPTION OF THE FIGURES

The accompanying figures illustrate non-limiting examples of the arrangement of the first and second regions within the locus.

Figure 1 – Second region forms a border around the perimeter of the first region
Figure 2 – Second region forms brackets either side of the first region
Figure 3 – Second region forms a series of horizontal or vertical strips amongst the first regions
Figure 4 – Second region forms a block within the first region
Figures 5 – 7 – First and second regions are adjacent
Figure 8 – 10 – A plurality of first and second regions present in mosaic patterns

CLAIMS

1. A locus at which insects feed comprising at least two regions, characterised in that:
   a) a first region comprises plants which produce at least a first insecticidal toxin; and
   b) a second region comprises plants which produce at least a second insecticidal toxin;

   wherein an insect which can develop resistance to the first toxin does not develop resistance to the second toxin, and the first region comprises plants which produce the first toxin but not the second toxin when the plants of the second region produce the second toxin but not the first toxin.

2. A locus according to claim 1 wherein the second region is within a quarter of a mile from the first region.

3. A locus according to claim 1 or 2 wherein the second region is adjacent to the first region.

4. A locus according to any of claims 1 to 3 wherein the second region is a border around the perimeter of the first region.

5. A locus according to claim 2 wherein the second region comprises one or more strips within the first region.

6. A locus according to claim 2 wherein a plurality of first and second regions are present in a mosaic pattern.

7. A locus according to claim 2 which comprises a random distribution of first and second regions.

8. A locus according to any of the preceding claims wherein the first insecticidal toxin has a different mode of action to the second insecticidal toxin.

9. A locus according to any of claims 1 to 7 wherein the first insecticidal toxin is a crystal protein from *Bacillus thuringiensis* and the second insecticidal toxin is a VIP protein from *Bacillus thuringiensis*, or vice versa.

10. A locus according to any of the preceding claims wherein the plants which comprise the first toxin and the plants which comprise the second toxin are from different genera.

11. A locus according to any of claims 1 to 10 wherein the plants which comprise the first toxin and the plants which comprise the second toxin are cotton plants.

12. A locus according to any of the preceding claims wherein at least 5% of the locus comprises the first region and least 5% of the locus comprises the second region.

13. A locus according to claim 12 wherein at least 20% of the locus comprises the first region and least 20% of the locus comprises the second region.

14. A method of controlling insects comprising providing a locus at which insects feed comprising at least two regions, characterised in that:
   a) a first region comprises plants which produce at least a first insecticidal toxin; and
   b) a second region comprises plants which produce at least a second insecticidal toxin;

   wherein an insect which can develop resistance to the first toxin does not develop resistance to the second toxin, and the first region comprises plants which produce the first toxin but not the second toxin when the plants of the second region produce the second toxin but not the first toxin.

15. Use of a locus according to any of claims 1 to 13 in a method of controlling insects.

16. A method of reducing the incidence of resistance of an insect to a first insecticidal toxin comprising the steps of providing a locus at which insects feed comprising at least two regions, characterised in that:

    a) a first region comprises plants which produce at least a first insecticidal toxin; and

    b) a second region comprises plants which produce at least a second insecticidal toxin;

wherein an insect which can develop resistance to the first toxin does not develop resistance to the second toxin, and the first region comprises plants which produce the first toxin but not the second when the plants of the second region produce the second toxin but not the first, so that insects which have developed or are developing resistance to the first insecticidal toxin are controlled by the second toxin.

17. Use of a locus according to any of claims 1 to 13 in a method of reducing the incidence of resistance of insects to a first insecticidal toxin.

18. A method according to claim 14 or 16 or a locus according to any of claims 1 to 13 wherein either the first or second region comprises Bollgard® cotton plants.

19. A method according to claim 14 or 16 or a locus according to any of claims 1 to 13 wherein the first region comprises Bollgard® cotton plants and the second region comprises VIP cotton plants, or vice versa.

IMPROVEMENTS IN OR RELATING TO ORGANIC COMPOUNDS

ABSTRACT

The present invention provides a method for reducing the incidence of resistance of insects to insecticidal plants. In particular, there is provided a locus at which insects feed comprising at least two regions, characterised in that: a) a first region comprises plants which produce at least a first insecticidal toxin; and b) a second region comprises plants which produce at least a second insecticidal toxin; wherein an insect which can develop resistance to the first toxin does not develop resistance to the second toxin, and the first region comprises plants which produce the first toxin but not the second toxin when the plants of the second region produce the second toxin but not the first toxin. The invention also provides a method for controlling insects.

FIGURE 1

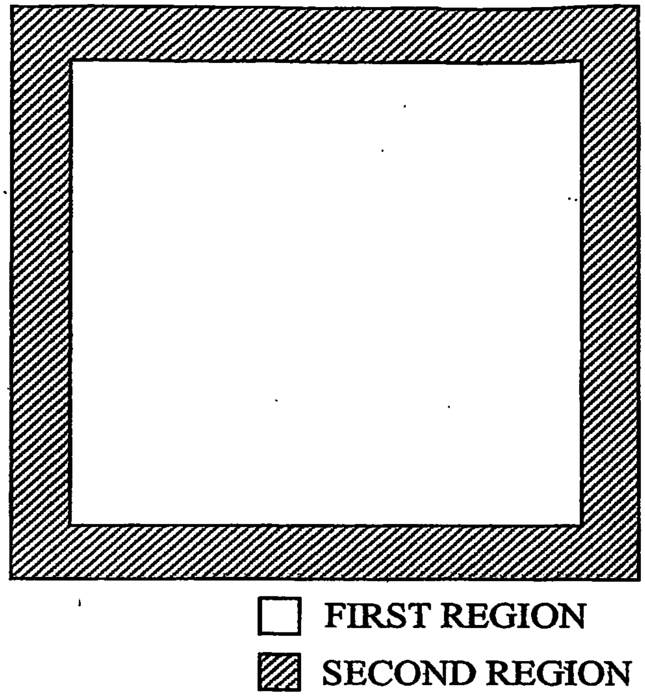

FIGURE 2

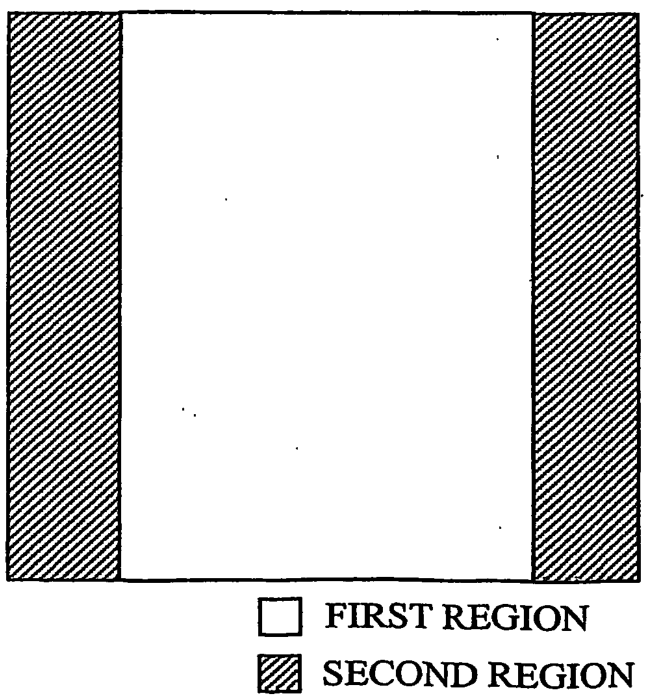

FIGURE 3

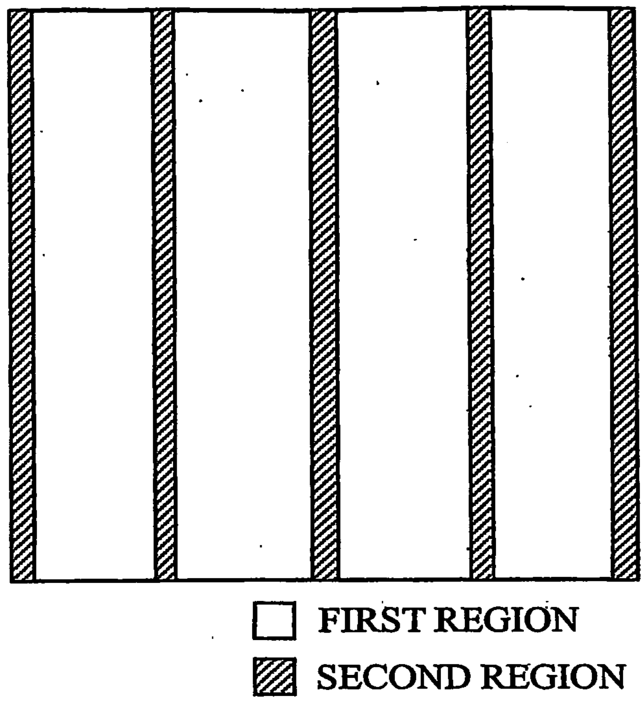

FIGURE 4

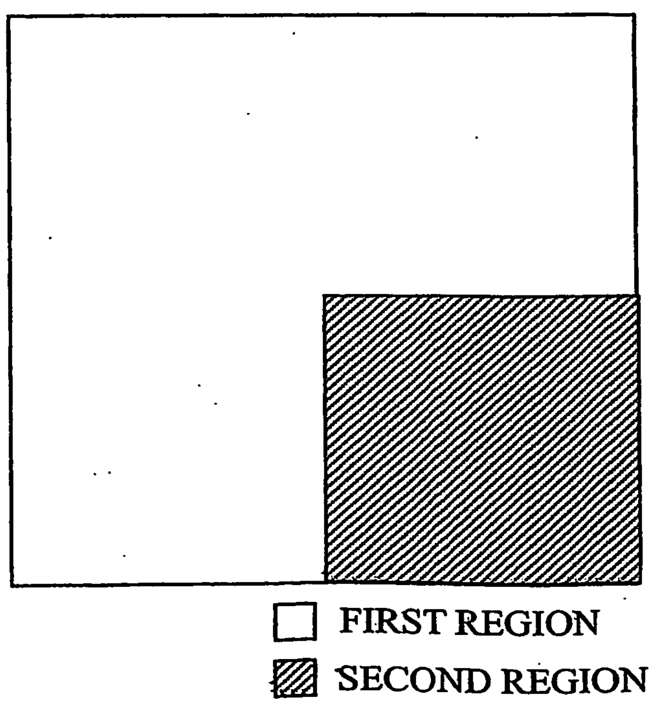

FIGURE 5

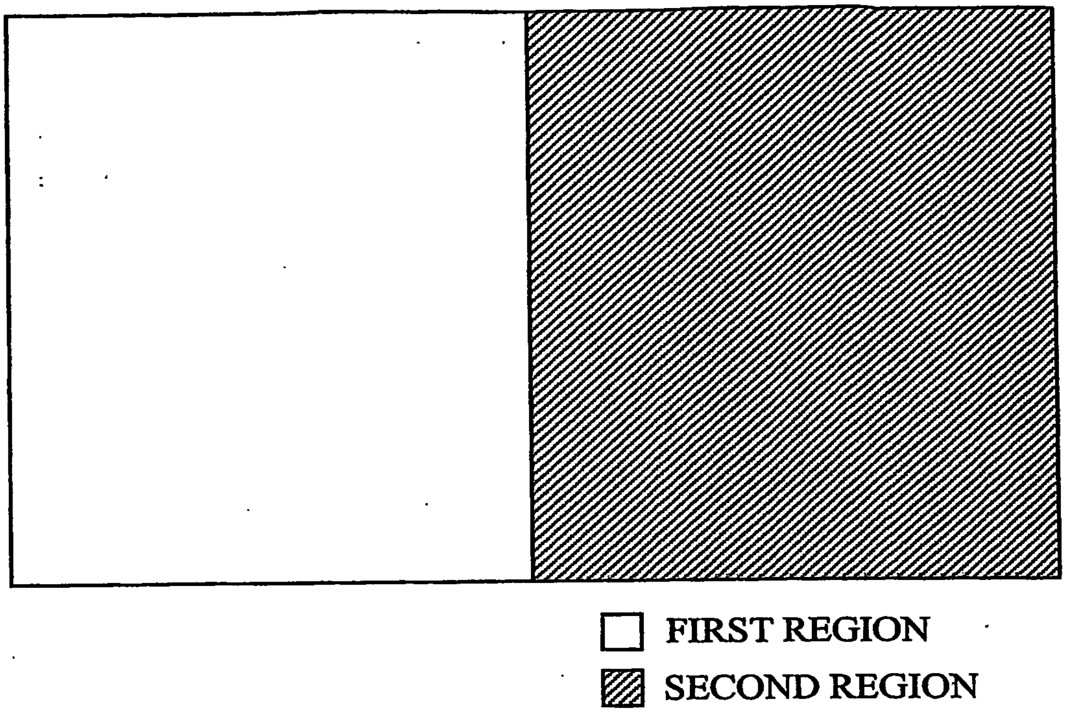

FIGURE 6

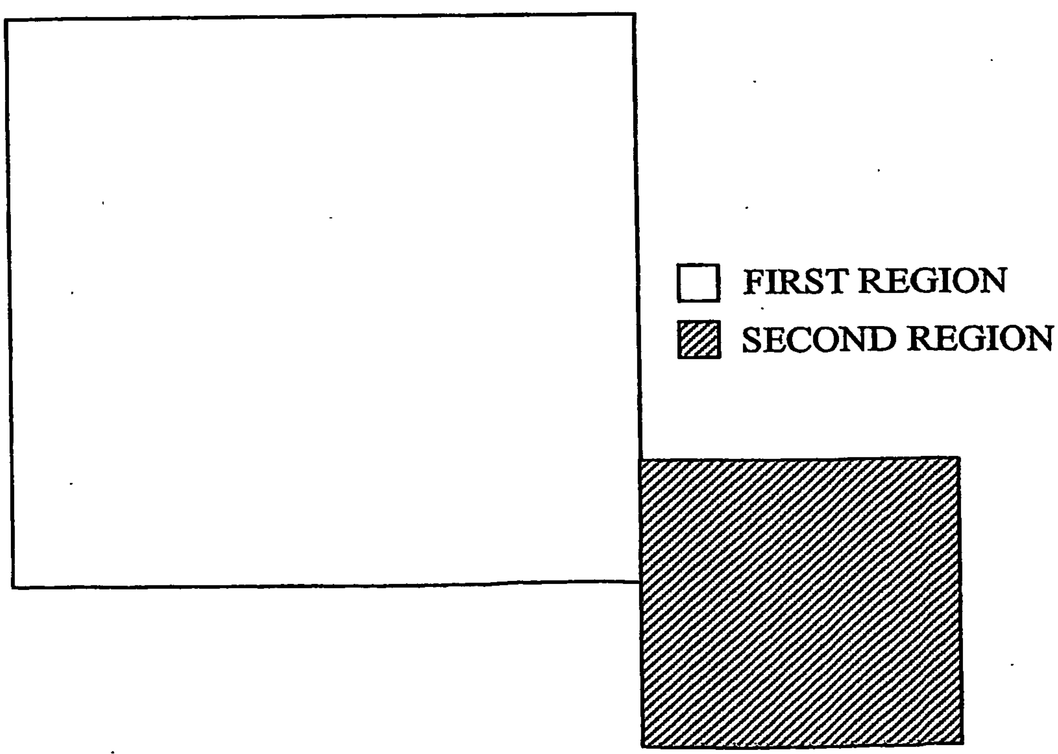

FIGURE 7

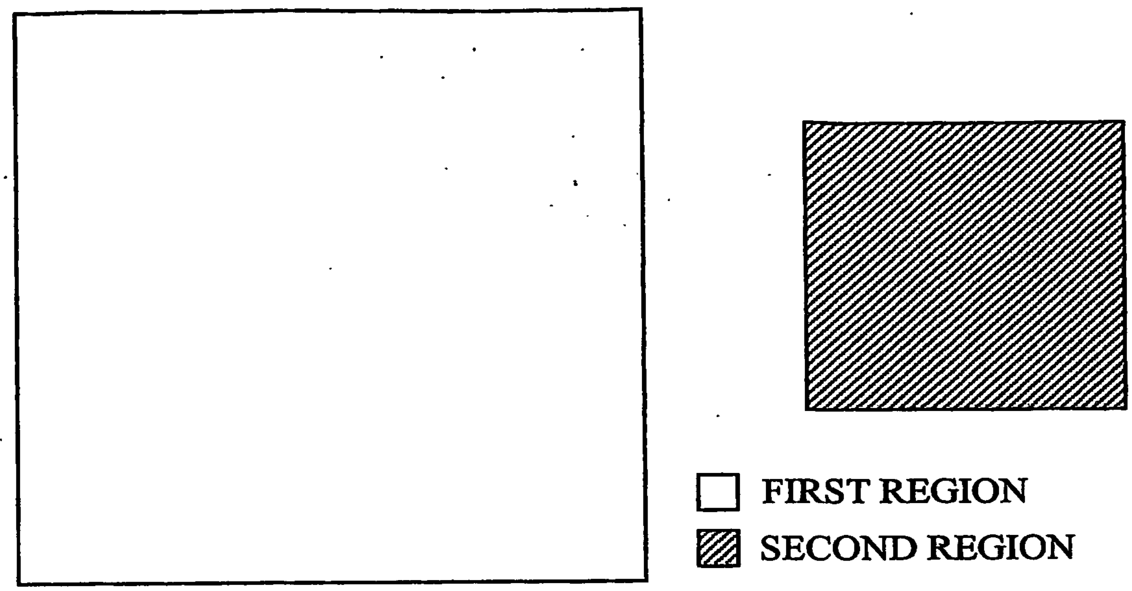

FIGURE 8

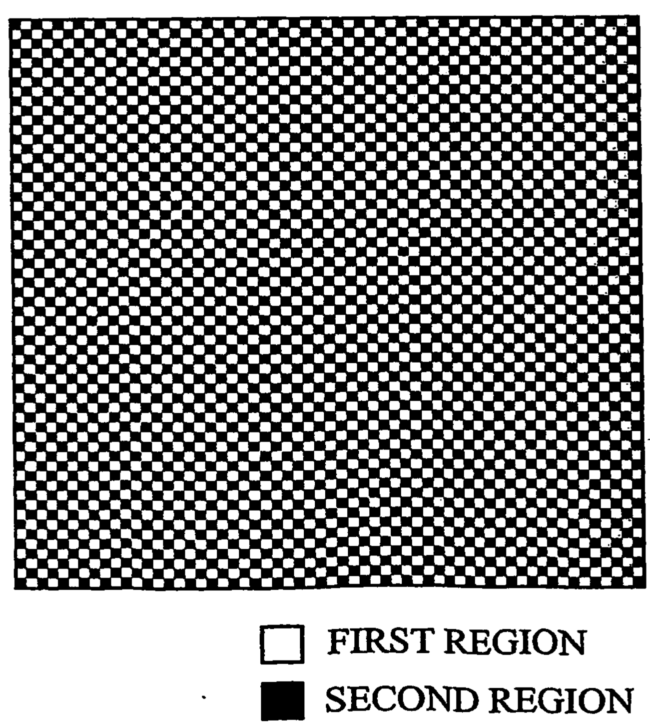

FIGURE 9

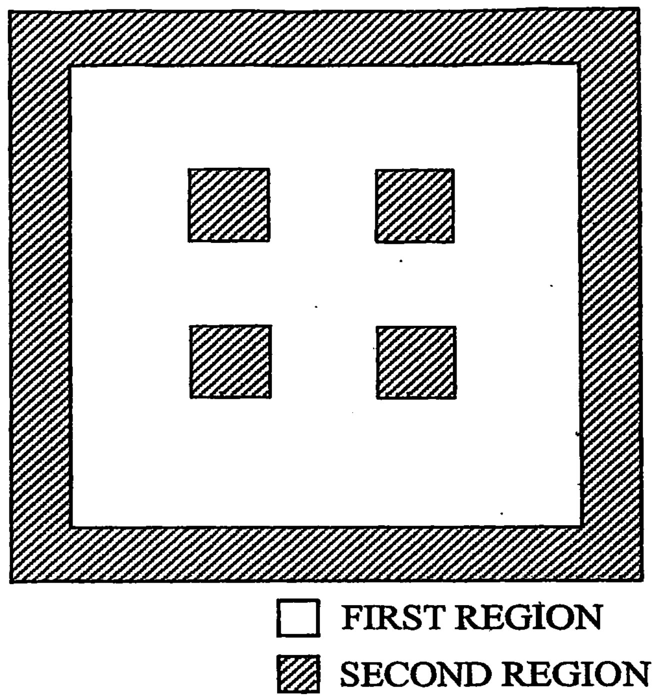

FIGURE 10